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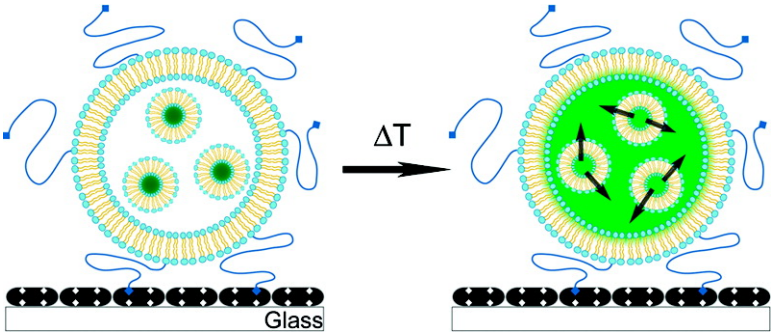

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Integrated Nanoreactor Systems: Triggering the Release and Mixing of Compounds Inside Single Vesicles

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Lipid vesicles constitute nanocontainer systems ideally suited for the isolation, preservation, transport, and localization of few[1] or single[2] molecules. Their ultrasmall dimensions (minimal diameters of 20 nm) allow unparalleled reduction of confined volumes to the zeptoliter range (1 zL = 10^{-21} L). The availability of lipids with variations in the hydrocarbon chains and the polar headgroups permits in addition the optimal design of a container that is tight and inert to the reactants and products of many biochemical processes like protein expression,[3] enzymatic reactions,[4] or mRNA transcription[5] to mention a few. The potential of these systems for miniaturization and bionanotechnology was nevertheless realized only after single vesicles were extracted from the *ensemble* and addressed as individuals, either by means of micromanipulation[6] or by directed assembly on patterned surfaces.[1,7] Here we present a method that allows the on-demand release and mixing of soluble compounds stored in the interior of individual vesicular nanoreactors.

Performing (bio)chemical reactions entails the controlled mixing of reactants. Inspired by cellular processes, we used self-assembly principles to create an integrated device capable of mixing zeptoliter to femtoliter volumes. The principle we employ is thermotropic permeability changes of lipid bilayers to polar solutes.[8] Maximum bilayer permeability (increase of several orders of magnitude) is reached at the characteristic ordered-fluid phase transition temperature (T_t) of the constituent lipid due to packing defects that create transient pores in the membrane.[9] As illustrated in Figure 1A, we created a nested system of vesicles composed of different lipids, having different T_t. This enabled us to define conditions under which small unilamellar vesicles (SUVs), trapped in the interior of a large unilamellar vesicle (LUV), release their cargo that is subsequently confined and mixed in the interior of the LUV.

The SUVs are composed of a 9:1 mixture of DPPC and DPPG.[10,11] They show low permeability for polar molecules below and above 41 °C (the T_t of both lipids[12]) allowing their convenient manipulation and storage at room temperature. Indeed, reactors stored for up to one week showed content-release properties identical to those of freshly made reactors. As a further precaution against leakage, we included the negatively charged lipid DPPG in the bilayer of the SUVs and the LUV. The resulting electrostatic repulsion keeps the SUVs "suspended" in the LUV suppressing interactions between themselves or the walls of the reactor that could cause uncontrolled leakage. The lipid composition of the LUVs[10] having a T_t of −18 °C guaranteed efficient confinement of the released polar solutes throughout the investigated temperature range.

To monitor the function of the reactor systems on an individual basis but in a parallel manner, we immobilized them on a solid support. We have described the prerequisite conditions for site-specific positioning of single vesicles elsewhere;[1] here LUVs are immobilized randomly on a glass substrate homogeneously coated with neutravidin.[13] The wide size distribution[14] of available LUVs

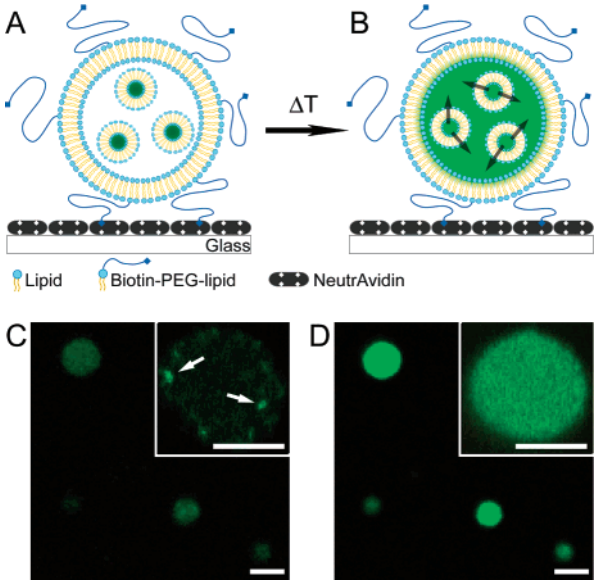

Figure 1. Principle of temperature-induced release and mixing of water-soluble molecules in the interior of immobilized vesicular reactors. (A) A large unilamellar vesicle (LUV) contains small unilamellar vesicles (SUVs) that are loaded with soluble reactants (high concentration of CF, quenched fluorescence = dark green). The LUV is immobilized via a biotin-PEG-lipid to a neutravidin covered surface. (B) Increasing the temperature (ΔT) across the ordered-fluid lipid phase transition of the SUVs results in the release (arrows) of their cargo, which is subsequently confined and mixed inside the LUV (dilution of CF, fluorescence increase = intense green). (C) Confocal fluorescence microscopy (LSM 510, Zeiss) of LUVs immobilized on glass. At a concentration of 15 mM, the fluorescence intensity of carboxyfluorescein (CF) inside the SUVs is low due to self-quenching. Here, intensity was digitally enhanced to enable vesicle visualization. Inset: higher magnification of a LUV allows identification of diffusing SUVs (white arrows). (D) Increase of the temperature from 25 to 45 °C causes release of the dye, which is then diluted. This is monitored as a sharp increase in the fluorescence intensity of all LUVs under observation. Inset: increase of fluorescence is uniform over the whole vesicle. All scale bars are 5 μm.

allowed simultaneous evaluation of the reaction process on different volume scales. A concentration of 0.3 mol % biotin−PEG−lipid[15] mediated a specific interaction of the LUVs with the neutravidin that is strong enough to allow long-lasting data acquisition but, due to the long PEG spacers, mobile enough to prevent vesicle destabilization or destruction.

As a proof of principle, we monitored the thermotropic phase transition-induced release of a fluorescent dye from SUVs into the lumen of immobilized individual LUVs. SUVs were first loaded with CF at a self-quenching concentration[12] and then entrapped in a larger vesicle.[11] Fluorescence confocal microscopy, Figure 1C, revealed a low average fluorescence intensity per immobilized LUV that originated from diffusing CF-loaded SUVs. Thermotropic

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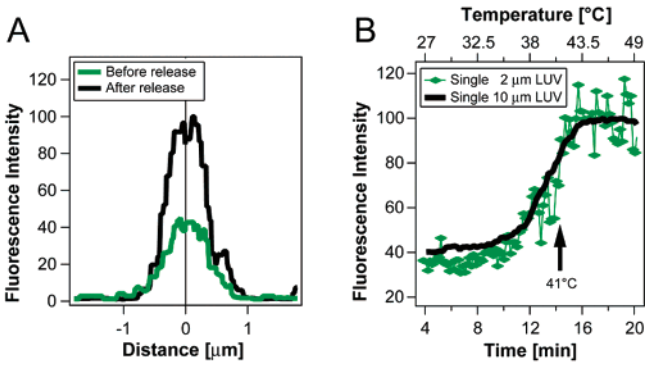

Figure 2. Thermotropic phase transition-induced release of CF inside LUVs. The values of both graphs were extracted from fluorescence confocal microscopy images. (A) Fluorescence intensity profile across a 2 μm diameter LUV at 25 °C (green) and after an increase of the temperature to 45 °C (black), 4 °C above the phase transition temperature of the lipid bilayer. (B) Time course of the increase of the mean fluorescence intensities of two single LUVs during a continuous temperature scan (1.5 °C/min). The point of inflection is very close to the $T_t = 41$ °C of the SUVs. During the temperature scan, the confocal plane had to be manually adjusted to compensate for the thermal expansion of the sample holder. Proper alignment is more critical for the 2 μm vesicle than for the 10 μm vesicle, resulting in an increase of experimental noise.

release and subsequent dilution of CF into the LUVs reduces self-quenching and increases fluorescence, Figure 1D.

The factor F of fluorescence intensity increase per reactor is a direct measure of the dilution ratio and depends mainly on (i) the number of encapsulated SUVs and (ii) the reproducibility of the release process. For a reliable reactor, one desires a small variation in *F*, i.e., in the final concentration of released reactants. Statistics over a few hundred LUVs (data not shown) revealed an almost ideal release process but also a variation of ±30% in the number of loaded SUVs per large vesicle, which, at the moment, is limiting the performance of the device. Figure 2A depicts a typical response of a single 2 μm diameter vesicle (total volume is 4 fL) with an $F = 2.5$ that corresponds to a 10-fold dilution of the released fluorophore.[16]

Figure 2B shows *F* for two different reactors (2 and 10 μm diameters) during a temperature scan through the ordered-fluid phase transition of the SUVs. The increase of the mean fluorescence is maximal at 41 °C, the T_t of the SUVs. All released compounds are retained in the reactor as shown by the stable baseline. The progressive temperature scan revealed a transition width of 3 °C and a half time of release of ~1 min. These results prove unambiguously that the self-quenched dye is released from the loaded SUVs at T_t but remains confined in the interior of the larger vesicle.

In summary, we have described a method that allows the parallel production of nanoreactor systems whose function is controlled by a remote stimulus. The reactors comprise a nested system of lipid vesicles, part of which release their content during a thermotropic phase transition. The integration of all components (reactants and products) in a single element eliminates the need for external manipulation/intervention[3−6,17] and therefore renders this nanoscopic system entirely autonomous. The smallest attainable size for vesicles (diameter ~20 nm, volume ~10^{-21} L) puts a lower limit to the total volume of the device of about 10^{-18} L, making it ideally suited for manipulating interacting partners at the single-molecule level. Using vesicles of several different phase transition temperatures, one can extend the process and realize a number of sequential reaction steps, each separated from the previous by about 3 °C. Combination of this method with vesicle-microarray[1] technology will permit the simultaneous observation and quantitative analysis of confined (bio)chemical reactions in thousands of separate reactors.

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References

(1) Stamou, D.; Duschl, C.; Delamarche, E.; Vogel, H. *Angew. Chem., Int. Ed.* **2003**, *42*, 5580.
(2) Rhoades, E.; Gussakovsky, E.; Haran, G. *Proc. Natl. Acad. Sci. U.S.A.* **2003**, *100*, 3197.
(3) Oberholzer, T.; Nierhaus, K. H.; Luisi, P. L. *Biochem. Bioph. Res. Co.* **1999**, *261*, 238.
(4) Walde, P.; Ichikawa, S. *Biomol. Eng.* **2001**, *18*, 143.
(5) Fischer, A.; Franco, A.; Oberholzer, T. *Chembiochem* **2002**, *3*, 409.
(6) (a) Chiu, D. T.; Wilson, C. F.; Ryttsen, F.; Stromberg, A.; Farre, C.; Karlsson, A.; Nordholm, S.; Gaggar, A.; Modi, B. P.; Moscho, A.; Garza-Lopez, R. A.; Orwar, O.; Zare, R. N. *Science* **1999**, *283*, 1892. (b) Karlsson, A.; Karlsson, R.; Karlsson, M.; Cans, A. S.; Stromberg, A.; Ryttsen, F.; Orwar, O. *Nature* **2001**, *409*, 150.
(7) (a) Michel, R.; Lussi, J. W.; Csucs, G.; Reviakine, I.; Danuser, G.; Ketterer, B.; Hubbell, J. A.; Textor, M.; Spencer, N. D. *Langmuir* **2002**, *18*, 3281. (b) Svedhem, S.; Pfeiffer, I.; Larsson, C.; Wingren, C.; Borrebaeck, C.; Hook, F. *Chembiochem* **2003**, *4*, 339. (c) Yoshina-Ishii, C.; Boxer, S. G. *J. Am. Chem. Soc.* **2003**, *125*, 3696.
(8) Papahadjopoulos, D.; Jacobson, K.; Nir, S.; Isac, T. *Biochim. Biophys. Acta* **1973**, *311*, 330.
(9) Van Hoogevest, P.; De Gier, J.; De Kruijff, B. *FEBS Lett.* **1984**, *171*, 160.
(10) Lipids dissolved in chloroform were mixed at different ratios, dried, and then rehydrated with the appropriate aqueous solution. We have used the following lipids (Avanti Polar Lipids): DPPC (1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine), DPPG (1,2-dipalmitoyl-*sn*-glycero-3-[phospho-*rac*-(1-glycerol)]), DOPG (1,2-dioleoyl-*sn*-glycero-3-[phosphor-*rac*-(1-glycerol)]), rhodamine-PE (1,2-dipalmitoyl-*sn*-glycero-3-phosphoethanolamine-*N*-(lissamine rhodamine B sulfonyl)), biotin−PEG−lipid (1,2-distearoyl-*sn*-glycero-3-phosphoethanolamine-*N*-[biotinyl(polyethylene glycol)$_{2000}$]). SUVs are made of DPPC/DPPG at a molar ratio of 9:1. LUVs are made of DOPG/rhodamine−PE/biotin−PEG−lipid, molar ratio 96:1:3.
(11) SUVs are hydrated with 15 mM CF (Sigma-Aldrich) and 200 mM sorbitol (Fluka). After 5 freeze−thaw cycles, the vesicle solution is extruded (Lipex Biomembrane, Inc.) eight times through filters of 400 nm pore diameter at 70 °C. The untrapped dye is removed by gel exclusion chromatography (Sephadex G-75) at room temperature, eluting with a solution of 10 mM KCl and 200 mM sorbitol. LUVs are prepared by hydrating with the solution of SUVs at room temperature for 2 days.
(12) Bramhall, J.; Hofmann, J.; Deguzman, R.; Montestruque, S.; Schell, R. *Biochemistry* **1987**, *26*, 6330.
(13) Microscope glass slides were cleaned (several detergent, water, and MeOH sonification steps) and incubated for 15 min with a solution of 0.1 mg/mL neutravidin (Pierce) in 2 mM NaH_2PO_4 and 200 mM sorbitol, pH 7.4. After being rinsed with buffer, the neutravidin surface was incubated for 5 min with a solution of LUVs and then washed again. The immobilized LUVs were imaged by laser scanning confocal fluorescence microscopy. Excitation at 488 nm, emission at LP 505 nm. The temperature of the solution was regulated from 20 to 50 °C with two Peltier elements; control and recording is implemented in LabVIEW (National Instruments).
(14) If desired, dialysis can be used to create an approximate lower limit to the size distribution.[18] An upper limit can be defined by gentle filtration through 5−10 μm pores, data not shown.
(15) Needham, D.; Kim, D. H. *Colloid Surf., B* **2000**, *18*, 183.
(16) A dilution calibration curve was recorded with a 36 well plate reader (Analyst 96.384, LJL BioSystems). The starting solution of 15 mM CF and 200 mM sorbitol was diluted with 2 mM NaH_2PO_4 and 200 mM sorbitol, pH 7.4. SUVs obtained by extrusion through 400 nm pore-size filters have a mean diameter of 250 nm, which corresponds to an average volume $V_{SUV} = 8 \times 10^{-18}$ L. The volume of a LUV with a diameter of 2 μm was $V_{LUV} = 4 \times 10^{-15}$ L. The average number of SUVs incorporated in one LUV in order to get a 10-fold dilution was ca. $V_{LUV}/(10 \times V_{SUV}) \approx 50$. From the concentration of SUVs, 10 nM, we would expect about 30 SUVs inside a 2 μm LUV. The actual number of SUVs per LUV was not measured, but this estimation gives two numbers that correlate well keeping in mind the significant distribution of vesicle sizes (±50%) and the variation in the loading of LUVs.
(17) Lei, G.; MacDonald, R. C. *Biophys. J.* **2003**, *85*, 1585. Velev, O. D.; Prevo, B. G.; Bhatt, K. H. *Nature* **2003**, *426*, 515.
(18) Schmidt, C.; Mayer, M.; Vogel, H. *Angew. Chem., Int. Ed.* **2000**, *39*, 3137.

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